Clinical Flow Cytometry Sample Preparation

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Overview

- Pre-Analytical considerations
  - Sample Type & Collection containers
  - Sample Transportation & Preservative media & Stability
- Sample Preparation
  - Environment: Lab temperature, humidity, light
  - Disaggregation, filter, wash, blocking
  - Different types of PBS, Lyse, intracytoplasmic reagents
  - LSW, SLW, SLNW
  - Diagnostic vs MRD applications
- Instrument settings
  - Speed, acquisition numbers, resolution
- Quality considerations

Flow Cytometry Guidelines

Cytometry Part B (Clinical Cytometry) 84B:286–290

Validation of Cell-based Fluorescence Assays: Practice Guidelines from the ICSH and ICCS – Part II – Preanalytical Issues

Bruce H. Davis,[1*] Amar Dasgupta,[2] Steven Kussick,[3] Jin-Yeong Han,[4] and Annalee Estrellado[5]; on behalf of ICSH/ICCS Working Group

bjh guideline

Guidelines on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms

Ulrika Johansson,[1] David Bloxham,[2] Stephen Couzens,[3] Jennifer Jesson,[4] Ricardo Morilla,[5] Wendy Erber,[6] Marion Macey[7] and British Committee for Standards in Haematology

H43-A2
Vol. 27 No. 11
Replaces H43-A
Vol. 18 No. 8

Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition

Handling, Storage, and Preparation of Human Tissues

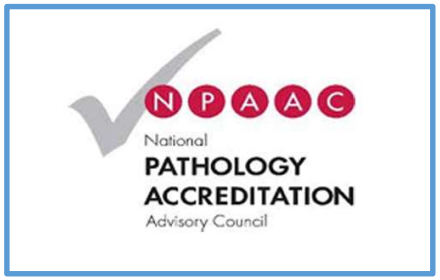
UNIT 5.2

Contributed by Lynn G. Dressler and Dan Visscher

Current Protocols in Cytometry (1997) 5.2.1-5.2.15

Regulatory Standards

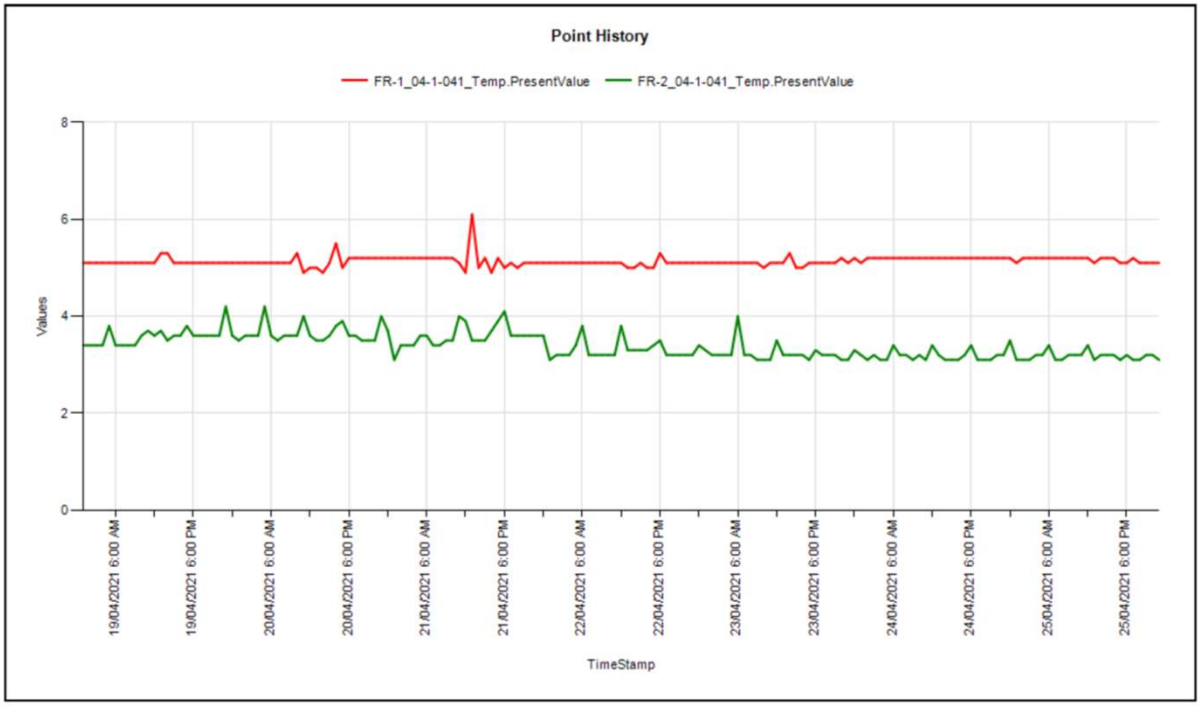
- Regulatory Requirements
  - Compliant to local regulatory bodies
    - NPAAC
    - NATA
    - TGA



- Minimum Patient Demographics
  - Name, UR#, Date of Birth
  - Address, Medicare #, date and time of collection

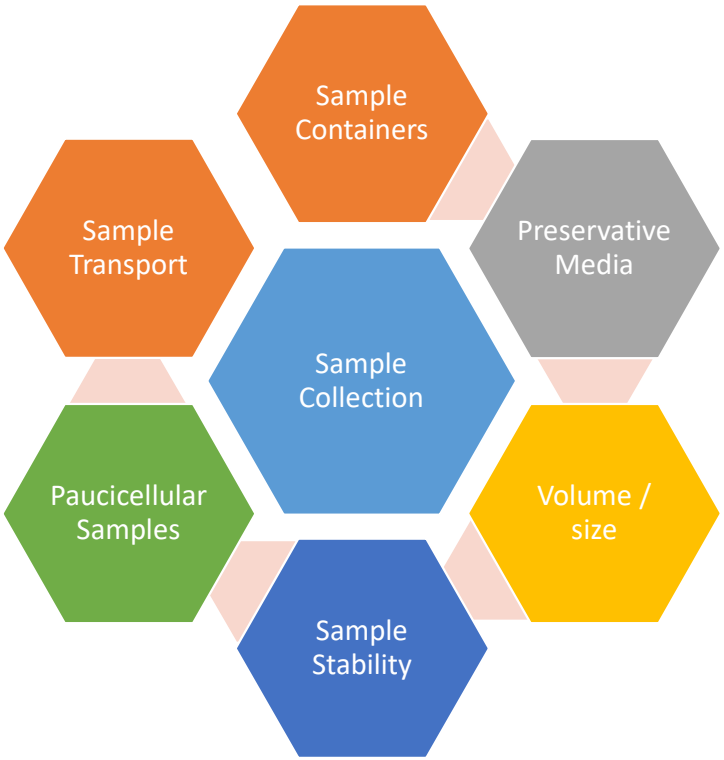
Regulatory Standards

- Facility Temperature and Humidity
  - Meeting reagent and consumable temperature specifications
  - Fridge and freezer temperature monitoring and alarm



Clinical Flow Sample Processing

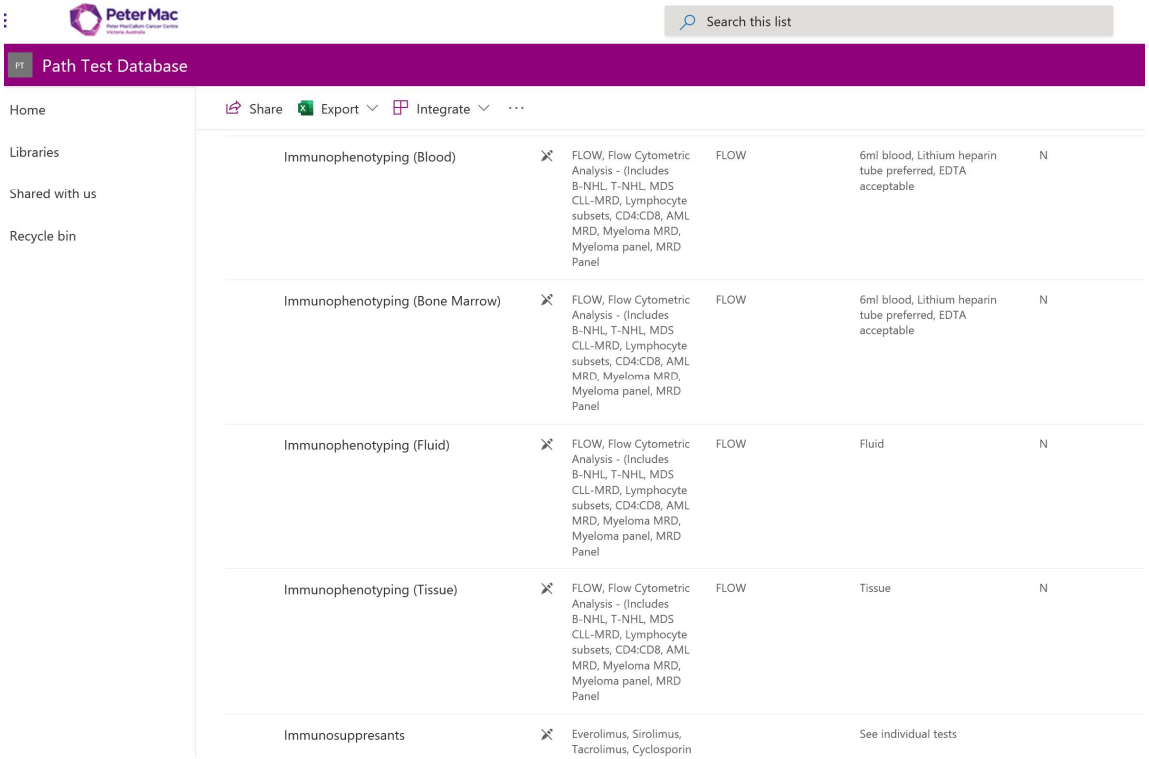
Pre Analytical



Analytical

Pre Analytical

- Pathology Test Database:
  - What tube, how many mLs, time, temperature, how many tubes.
  - Expedite sample, minimize disruption and save time for lab.



Pre Analytical

- **Minimum sample requirements**
  - For peripheral blood and bone marrow, 1-4mL of specimen collected in Lithium Heparin or EDTA is required
  - For solid tissue and body fluids, the exact minimum volume required will depend on the cell count.
  - In general, a minimum of 1ml of prepared cell preparation with a white cell count of > 1.0 $x10^9/L$ is required to perform a comprehensive lymphoproliferative panel investigation

Sample Types

- Flow Cytometric assessment relies on the availability of a suitable viable specimen:
  - Peripheral blood (PB)
  - Bone Marrow Aspirate (BMA)
  - Lymph Node (LN)
  - Tissue biopsy
  - Fluids (pleural, ascites, FNA, CSF etc..)
  - Cryopreserved

Collection Containers

Lithium/Sodium Heparin

ACD

EDTA

Pots

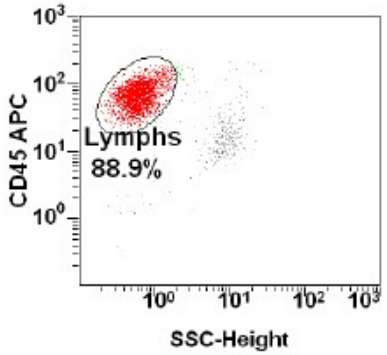
CSF Tubes

Sample Stability

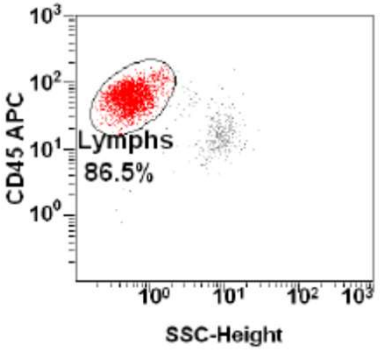
- Variables affecting specimen integrity, which are a composite of:
  - Anticoagulant & time
    - EDTA, Na/Li Heparin, ACD
    - Aged artefacts
  - Temperature
    - Recommended to keep samples at room temp (18-22C)
    - Temp tracking devices for long journeys
  - Stabilization material
    - Samples requiring long journeys to testing site
    - Volatile cells, ie CSF, PCs
  - Preparation methodology
    - Bulk lysis vs post-lysis
  - Varies depending on interrogated population
    - Specimen viability differs for lymphocytes, blasts and plasma cells.

Lymphocyte Stability

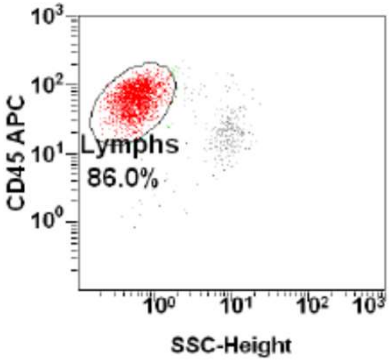
CLL – RT no RPMI



0 hours



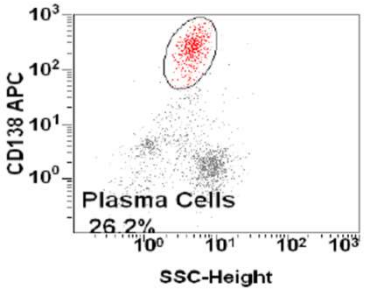
24 hours



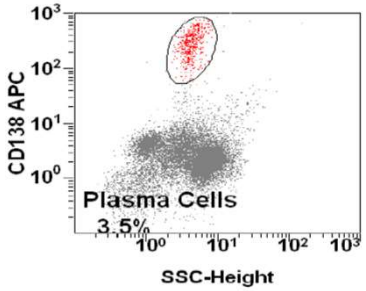
48 hours

Plasma Cell Stability

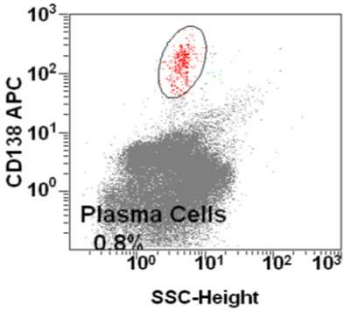
MM – RT no RPMI



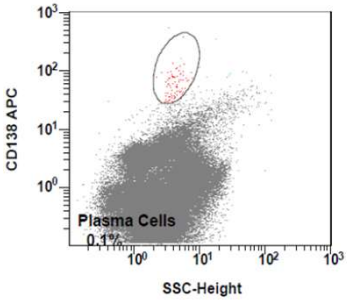
0 HRS: 26.2%



24 HRS: 3.5%



48 HRS: 0.8%



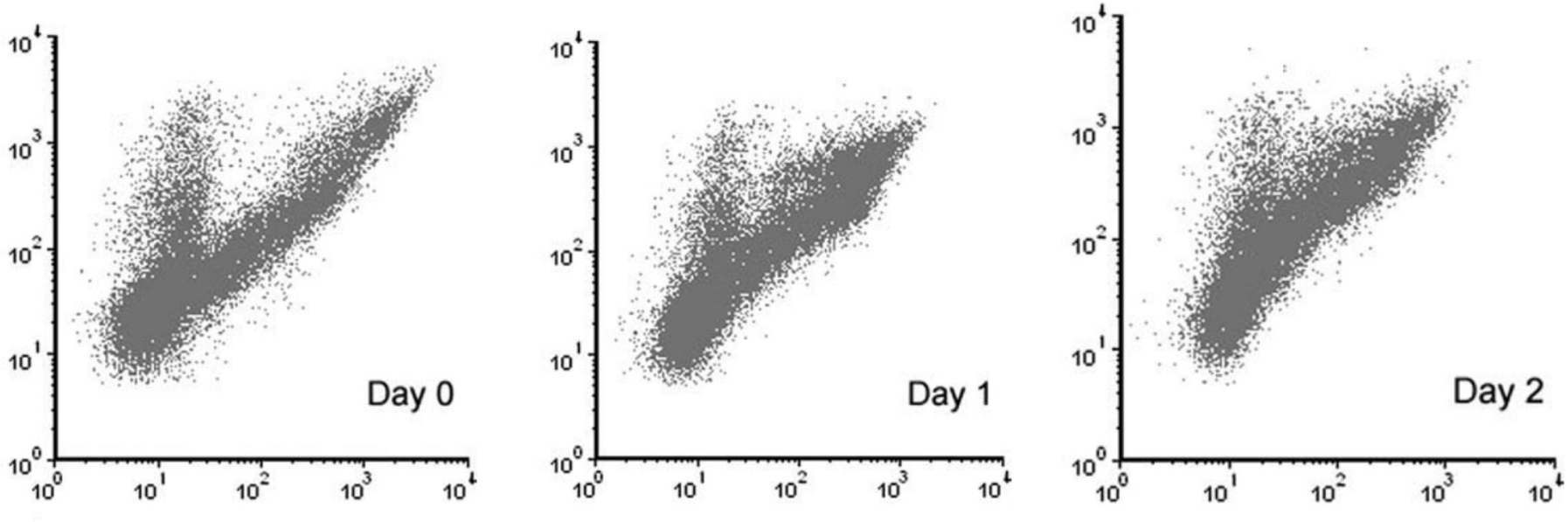
72 HRS: 0.1%

Cytometry Part B (Clinical Cytometry) 82B:217–228 (2012)

Original Article

Altered Neutrophil Maturation Patterns that Limit Identification of Myelodysplastic Syndromes

Sara A. Monaghan,[1*] Urvashi Surti,[1,2] Ketah Doty,[1] and Fiona E. Craig[1]



Monaghan et el. Cytometry B 2012

Sample Collection Tips

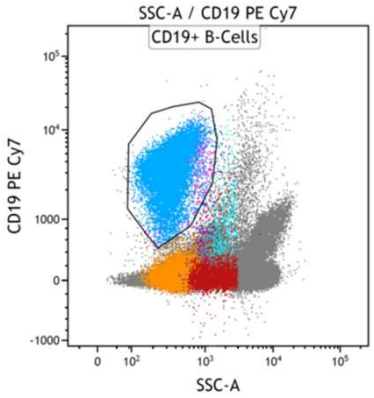
- Peripheral Blood venepuncture:
  - Improper site or prolonged tourniquet can result in haemolysis
  - Avoid clotted sample

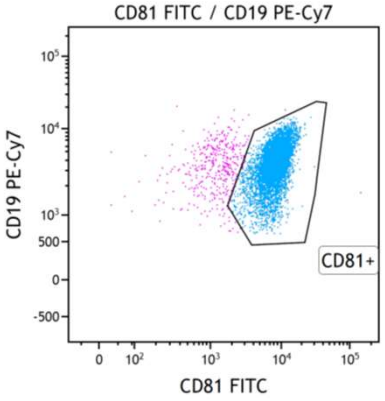
- Bone Marrow Aspirate:
  - Ideally use first draw
  - Subsequent draws are haemodilute
  - Confirm representation with mast cells, haematogones and progenitor cells.

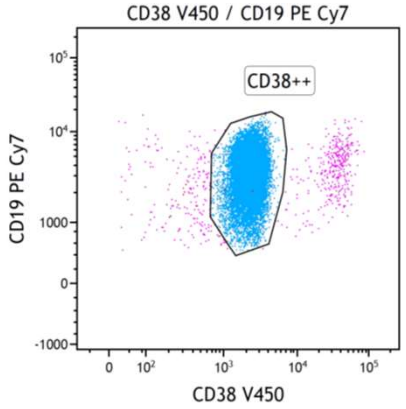
Bone Marrow Aspirate Representation

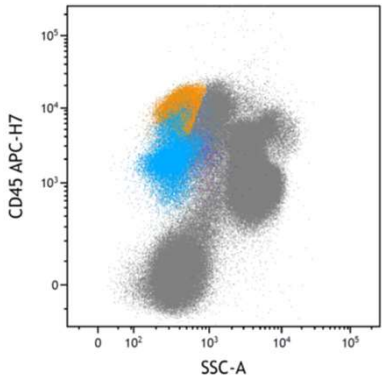
Multiple Myeloma Panel

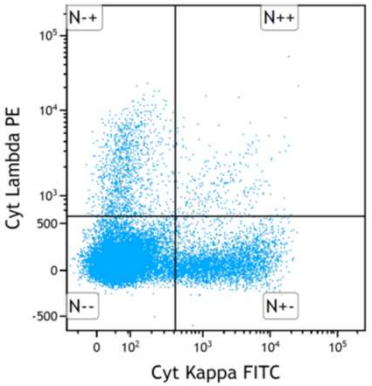
B-cell Precursors



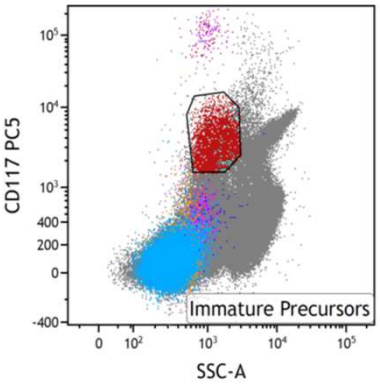


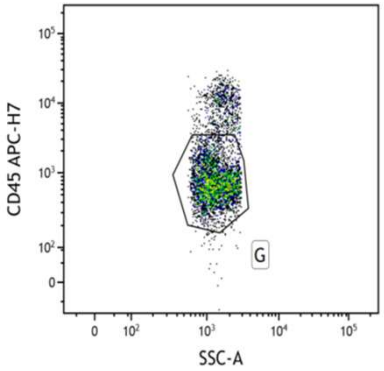




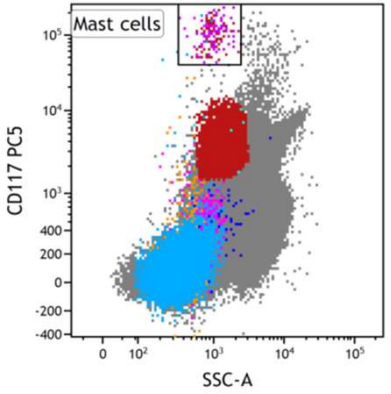


Immature Myeloid Precursors





Mast Cells



Sample Collection Tips

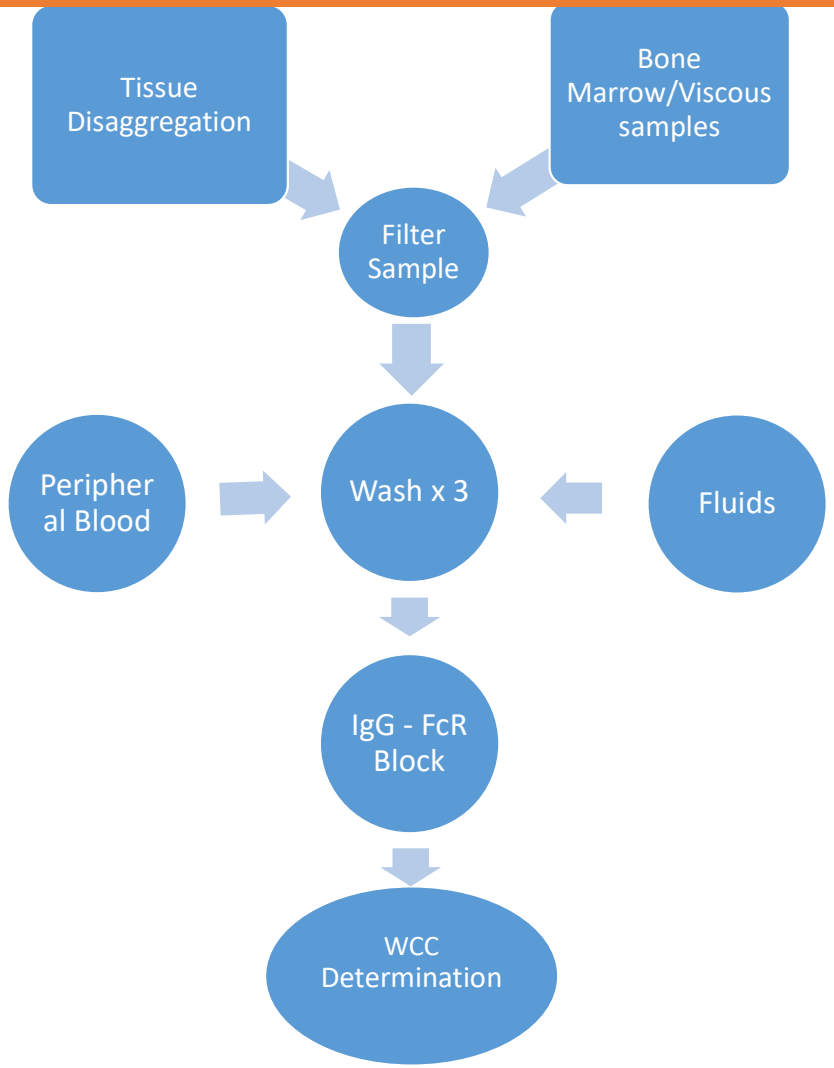
- Tissues:
  - Do not place core biopsies in surgical gauze, in saline is adequate
  - Add preservative media where appropriate (add RPMI upon arrival)
- Fluids (peritoneal, pleural, ascites, FNA etc):
  - Variable cellular concentration
  - Sufficient volume/concentration (ideally >10mLs)
  - CSF and BAL > 1mL atleast, the more the better
    - Pool samples from other departments
  - Add preservative media where appropriate:
    - CSF specific collection tubes to increase viability and recovery
  - Paucicellular strategy:
    - [ ]300uL → stain 200uL
    - 100uL add on

Analytical – Sample Processing

- Triage and labelling
  - Ascertain question being asked
  - Patient history & clinical notes
  - Set up appropriate panels to find answer

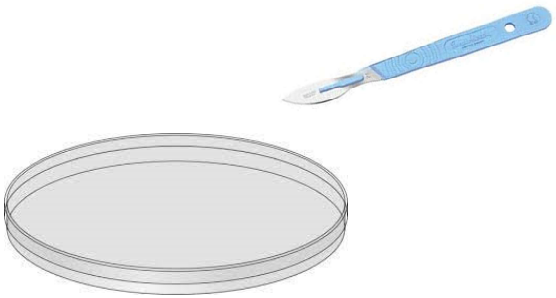
- System to ensure daughter tubes are auditable and traceable

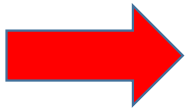
Sample Processing



Sample Processing

- Disaggregation of tissue
  - Can use mechanical splicing or enzyme digestion
  - Ensure tissue is cut in all facets and angle
    - Attention to abnormal macroscopic areas
  - Avoid coloured markings





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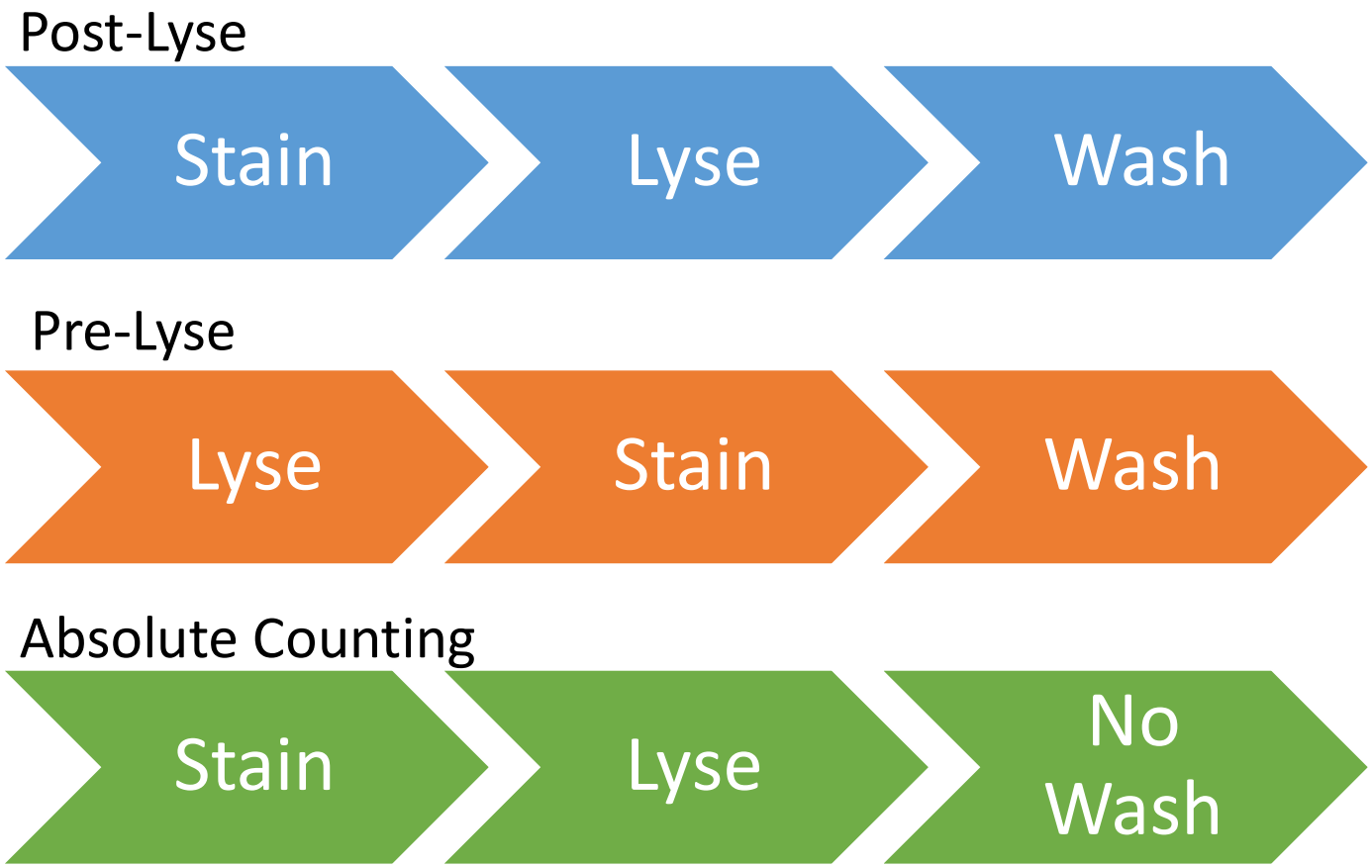
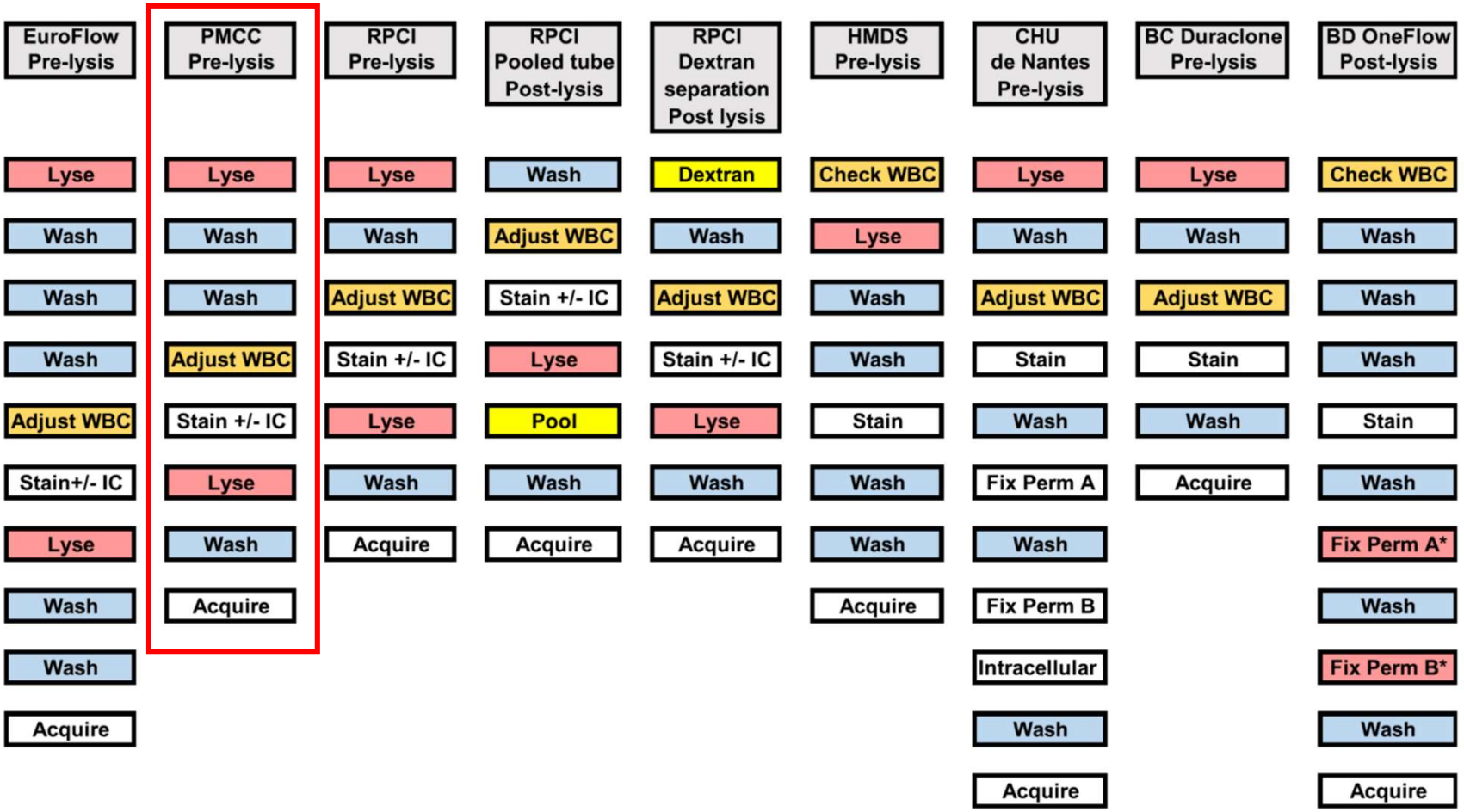
Analytical Procedures



Pre-Lysis Methodologies



Maclachlan et al. Pathology 2021

Analytical Procedures

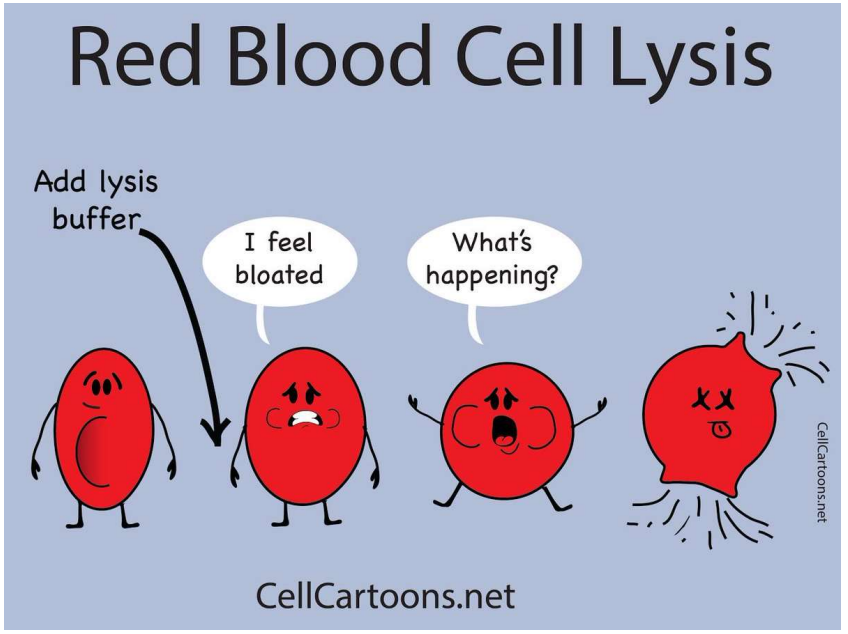
- **Interferences**
  - The principal interference is from un-lysed red blood cells which can interfere with the gating of the cell population of interest. This occurs most commonly in samples with significant numbers of nucleated red blood cells. Target cells may also prove resistant to lysis.
  - Non-specific uptake of antibodies by Fc receptors may contribute to the background noise.

Sample Processing Tips

- Washing:
  - Decant (risk of losing cells if not dry button) vs aspirate (slow but steady)
    - Mechanical aspirator if dealing with large volumes
  - Better separation of light chains – increase volume of PBS using 15ml tubes
    - Thoroughly mix sample through PBS
- Blocking:
  - Immunoglobulin to block FcR binding non-specifically to antibodies.
- Staining:
  - Avoid blood sticking to the side of tubes
  - Dispense full amount of antibodies, can result in reduction in MFI if not.
  - Vortex very well, especially for dried/lyophilised down reagents
- Intracytoplasmic staining:
  - Ensure dry button after initial fixation step
  - Dislodge button adequately before adding cytoplasmic antibodies.

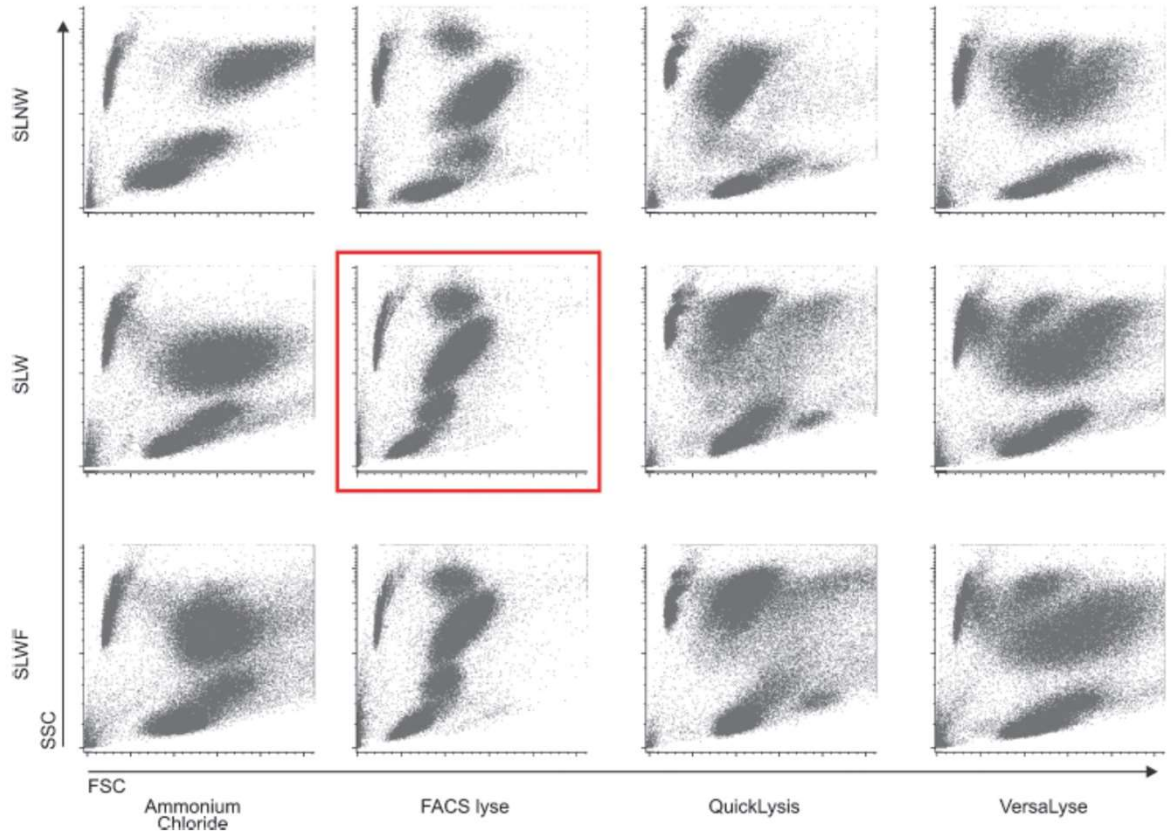
Sample Processing

- Lysing:
  - Atleast 10 minutes for complete lysis of RBCs
  - Prolonged lysis may affect WBCs
  - Prelysis – Adding 10mLs at a time and mix, yields better lysed RBC than adding all at once
  - Target cells and nRBCs are resistant



Sample Processing

- Different types of Lyse solutions: fixative and fixative free
  - Impact on physical and fluorescent patterns



Orfao et al. Leukemia 2012.

Other Processing Methods

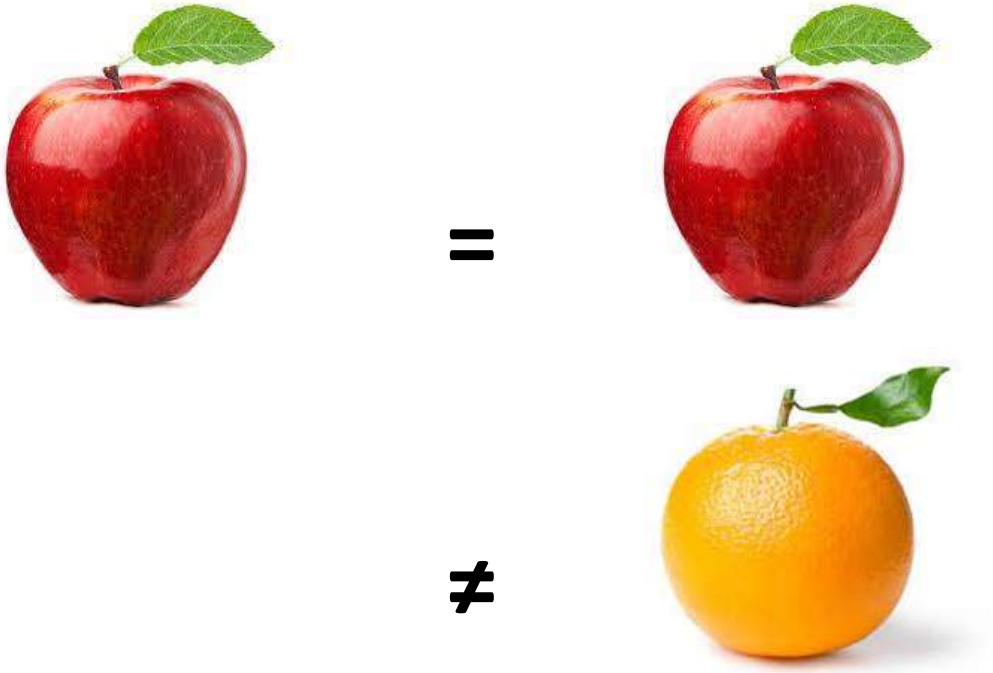
- Ficol-Paque density gradient separation
  - Less frequently performed in diagnostic labs
  - No literature evidence of false negative results or patients having clinically inferior outcomes compared to other methods
  - More manipulation, more opportunities to alter cellular characteristics.
- Magnetic beads separation
  - Purify specific populations (positively or negatively select) for clinical trials

Sample Acquisition

- Vortex sample before acquisition
  - Keep in dark if not run immediately
  - Keep at 4C if not acquired same day
- Setup appropriate gating strategy
- Determine stop gate and stop count (denominator)
- Acquisition speed (coincidence events and data resolution)

Quality Control – Antibody Pre-Accepetance Testing

- Assess integrity of antibody has not been compromised during transportation process



Antibody Cocktail Testing

- Assess integrity of antibody cocktail after reconstitution
  - Performance of individual antibody consistent
  - MFI and % within acceptance limits

Current

=

New

Laboratory Quality Assurance

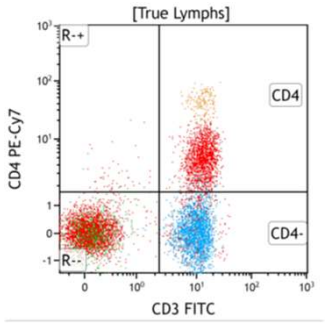
- Assay process controls
- Staff Competency assessment
  - Consensus to improve lab practices
- Continuing professional development
- SOPs
- Training Records
- Professional Certification
- EQAP/proficiency programs

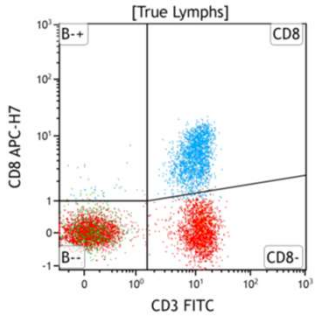
Sample Quality Assessment

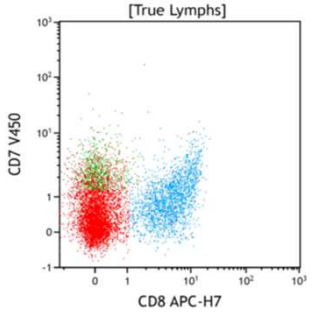
Physiological vs Technical artefact :

- Fsc vs Ssc
- Non-specific binding
- Loss CD4/7 expression aged sample
- Population loss
- Technical artefact: no or incorrect antibody added, instrument blockages, tandem dye decoupling

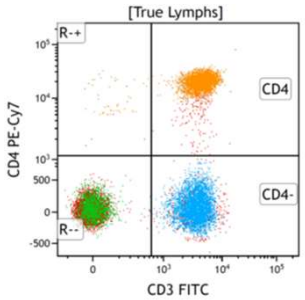
Old Sample

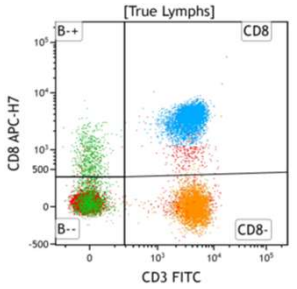


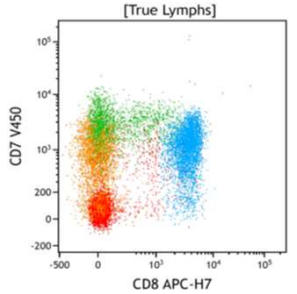




New Sample







Thank you!